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Patch Clamp Studies on Root Cell Vacuoles of a Salt-Tolerant and a Salt-Sensitive *Plantago* Species[1]

Regulation of Channel Activity by Salt Stress

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ABSTRACT

***Plantago media* L. and *Plantago maritima* L. differ in their strategy toward salt stress, a major difference being the uptake and distribution of ions. Patch clamp techniques were applied to root cell vacuoles to study the tonoplast channel characteristics. In both species the major channel found was a 60 to 70 picosiemens channel with a low ion selectivity. The conductance of this channel for Na^+ was the same as for K^+, $P_{K^+}/P_{Na^+} = 1$, whereas the cation/anion selectivity (P_{K^+}/P_{Cl^-}) was about 5. Gating characteristics were voltage and calcium dependent. An additional smaller channel of 25 picosiemens was present in *P. maritima*. In the whole vacuole configuration, the summation of the single channel currents resulted in slowly activated inward currents ($t^{1/2}$ = 1.2 second). Inwardly directed, ATP-dependent currents could be measured against a ΔpH gradient of 1.5 units over the tonoplast. This observation strongly indicated the physiological intactness of the used vacuoles. The open probability of the tonoplast channels dramatically decreased when plants were grown on NaCl, although single channel conductance and selectivity were not altered.**

Plant cell membranes contain ion channels, which form pathways for passive, *i.e.* thermodynamically downhill, ion movement. These channels have different specific functions: they play a role in cation transport, *e.g.* K^+ in guard cells (18), dissipation of electrical gradients in chloroplasts (17), and energy storage in vacuoles by malate accumulation (1). Apart from channels with a specialized function, in several plant species a class of tonoplast channels has been identified with a less obvious specific function. Their importance probably lay in turgor regulation and charge balance between vacuole and cytoplasm (11, 12).

Passive transport through channels, together with primary tonoplast pumps, may also be involved in selective uptake and excretion, of ions playing a role in salt tolerance (10, 16, 19). Many salt tolerant plant species sequester harmful ions, such as Na^+ and Cl^-, in vacuoles (5, 8). The loading of the vacuole with Na^+ from the cytoplasm is probably driven by the ΔpH via antiporters (6, 7, 15), while downhill unloading can proceed through ion channels. The ΔpH is generated by the tonoplast ATPase, and possibly the PPase, which also builds up the electrical tonoplast potential, driving passive fluxes through the channels. This implies that regulation of the tonoplast ATPase (9, 10) must take place under salt stress conditions. Regulation of vacuolar ion channels and ATPases can prevent toxic ions from reentering the cytoplasm. For that purpose ion channel selectivity, gating characteristics, and numbers of channels per area may alter in order to create a better defence mechanism against a high influx of NaCl. Also, ATPase proton currents over the tonoplast might be altered.

Electrophysiological experiments with intracellular microelectrodes, showed a difference in K^+/Na^+ uptake selectivity ratio between *Plantago maritima* and *Plantago media*. The uptake selectivity ratio, defined as (uptake $K^+/[K^+]_{out}$)/(uptake $Na^+/[Na^+]_{out}$), depended on the presence of NaCl during growth. In the absence of NaCl it was about 4.0 for *P. media* and 1.7 for *P. maritima* (2, 14).

The present study was done in order to find out how differences between the two species in uptake selectivity, ion distribution, and membrane conductance in intact roots are related to single channel properties of the tonoplast. It was also done to investigate whether changes in these characteristics occurred after induction of NaCl stress.

MATERIALS AND METHODS

Plant Material

Seeds of *Plantago maritima* L. and *Plantago media* L. were germinated on vermiculite. Seedlings 14 d old were transferred to climate chambers with the following regime: a 12 h light period, 58 W m^{-2}, 60% RH, day and night temperature of 20 and 18°C, respectively. Plants were grown in 30 L containers on 25% Hoagland nutrient solution (2). The solution was renewed twice a week and supplemented with 0, 25, or 100 mM NaCl. Addition of NaCl started in the fourth week after germination, and the final concentrations were reached in 1 (25 mM) or 1.5 weeks (100 mM).

Isolation of Vacuoles

Roots with a diameter of 1 to 1.5 mm were cut in thin slices with a sharp razor blade, while immersed in the experimental solution. Vacuoles, ranging from 5 to 50 μm in diameter, were spontaneously freed from the tissue and transferred to the experimental chamber (1).

[1] Ecotrans publication No. 15.

Experimental Solutions

Viability and tonoplast ATPase activity were tested in experiments, using the whole vacuole configuration. ATP was added to the bath solution and the inwardly directed current was measured. In these experiments the bath solution had the following composition: 25 mM Mes/Tris (pH 7.4), 1 mM $CaCl_2$, 50 mM KCl, and 1 mM $MgCl_2$. The high Ca^{2+} concentration was chosen to assure a good seal between electrode and membrane; Mg^{2+} was added to stimulate the ATPase activity. The pipette solution was: 25 mM Mes/Tris (pH 5.9), 50 mM KCl, and 1 mM EGTA. AΔpH of 1.5 units (medium minus vacuole) was chosen to test the capability of the ATPase pump to generate a current against a proton gradient. A relatively high buffer concentration was used to maintain the proton gradient. EGTA was used to lower the free Ca^{2+} in the pipette, and thereby prevent the ruptured membrane from resealing, after a whole vacuole configuration was established. In most other experiments, the bath and pipette solutions had the following composition: 10 mM Mes/Tris (pH 6.9), 0.1 mM $CaCl_2$, and 100 mM KCl. In all solutions the osmolarity was adjusted with sorbitol to 550, 600, and 680 mOsmol for plants grown on Hoagland, supplemented with 0, 25, and 100 mM NaCl, respectively.

Electrophysiology

Electrodes were prepared from soft glass capillaries; they were pulled on a two-stage puller, coated with Sylgard, and heat-polished. Resistances varied between 2 and 10 Mohm depending on the pipette geometry and the used solution.

Giga ohm seals between electrode and tonoplast were made by gentle suction and usually appeared within a second. The whole vacuole configuration was made with 5 ms pulses of 600 to 1000 mV. Voltage clamp and current clamp measurements were done with a List Electronics EPC 7 amplifier. Capacity compensation and series resistance compensation (whole vacuole configuration) were done with circuitry on the amplifier. Pulses and data were transferred via a CED 1401 A/D converter, under control of 'patch clamp' software (CED, Cambridge, UK). Single channel data were filtered at 100 to 500 Hz (8 pole Butterworth characteristics), and whole vacuole measurements at 1 to 100 Hz. Data were sampled at 1 to 10 kHz on a hard disc combined with a tape streamer backup system. Analysis for open/closed time and amplitude distributions were done with the above-mentioned software.

RESULTS

Tonoplast ATPase Activity

It was tested whether the isolated vacuoles were still physiologically active. To this end the ATP-dependent, inwardly directed currents were measured in the whole vacuole configuration. To establish the ability of the tonoplast ATPase to generate a current against a proton gradient, a ΔpH (bath solution minus vacuole) of 1.5 units was applied, while the tonoplast potential was clamped at 0 mV. Average vacuoles, obtained from either species whether grown with or without NaCl, were able to pump 30 to 50 pA[2] currents into the vacuole after addition of 4 mM ATP (Fig. 1). After switching to current clamp (I = 0), potentials were 20 to 30 mV inside positive; this means that a protonmotive force of at least 110 to 120 mV could be generated.

[2] Abbreviation: pA, picoampere; pS, picosiemens.

Single Channel Conductance and Selectivity

Figure 2 shows results of experiments with excised, inside out patches. Recordings are shown of tonoplast channels of *P. maritima* and *P. media* and their voltage current characteristics. Symmetrical solutions, containing 100 mM KCl, were used. Tonoplast patches of both species exhibited 60 to 70 pS channels which were voltage dependent. In *P. maritima* a second channel was observed, with a conductance of 25 pS, which appeared less often. Also in *P. media* a low conductance channel was sometimes present although with an even lower frequency (results not shown).

For activation, all these channels needed a hyperpolarized tonoplast potential, vacuolar side negative, and a high cytoplasmic Ca^{2+} concentration. Using an outside out patch configuration, the bath solution was titrated with EGTA to remove free Ca^{2+}. Channel activity was thereby reduced to zero when the Ca^{2+} concentration dropped below 1 μM (results not shown).

To determine the K^+/Na^+ selectivity, KCl in the bath solution was replaced by NaCl. Channel conductances remained the same as did the reversal potential, indicating a P_{K^+}/P_{Na^+} of 1. The K^+/Cl^- selectivity was determined using excised patches in asymmetrical KCl or NaCl solutions (10 mM in the bath, 100 mM in the pipette). A reversal potential of -27 to -30 mV was found, in both *P. maritima* and *P. media*. In double pulse (1) experiments with whole vacuoles (Fig. 3, a and b), the same reversal potential was observed from the tail current direction, showing that whole vacuole

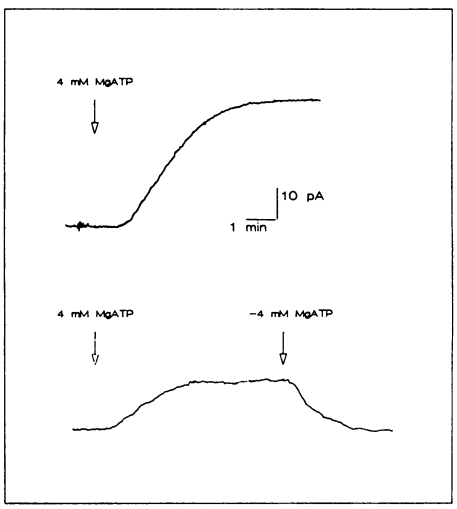

Figure 1. ATP dependent inward currents induced by 4 mM MgATP added to the bath. Whole vacuole configuration. Pipette solution: 50 mM KCl, 25 mM Mes/Tris (pH 5.9), 1 mM EGTA. Bath solution: 50 mM KCl, 25 mM Mes/Tris (pH 7.4), 1 mM $CaCl_2$, 1 mM $MgCl_2$. Upper trace: *P. media* grown without NaCl. Vacuole diameter was 35 μm. Lower trace: *P. media* grown with 100 mM NaCl. Vacuole diameter was 28 μm.

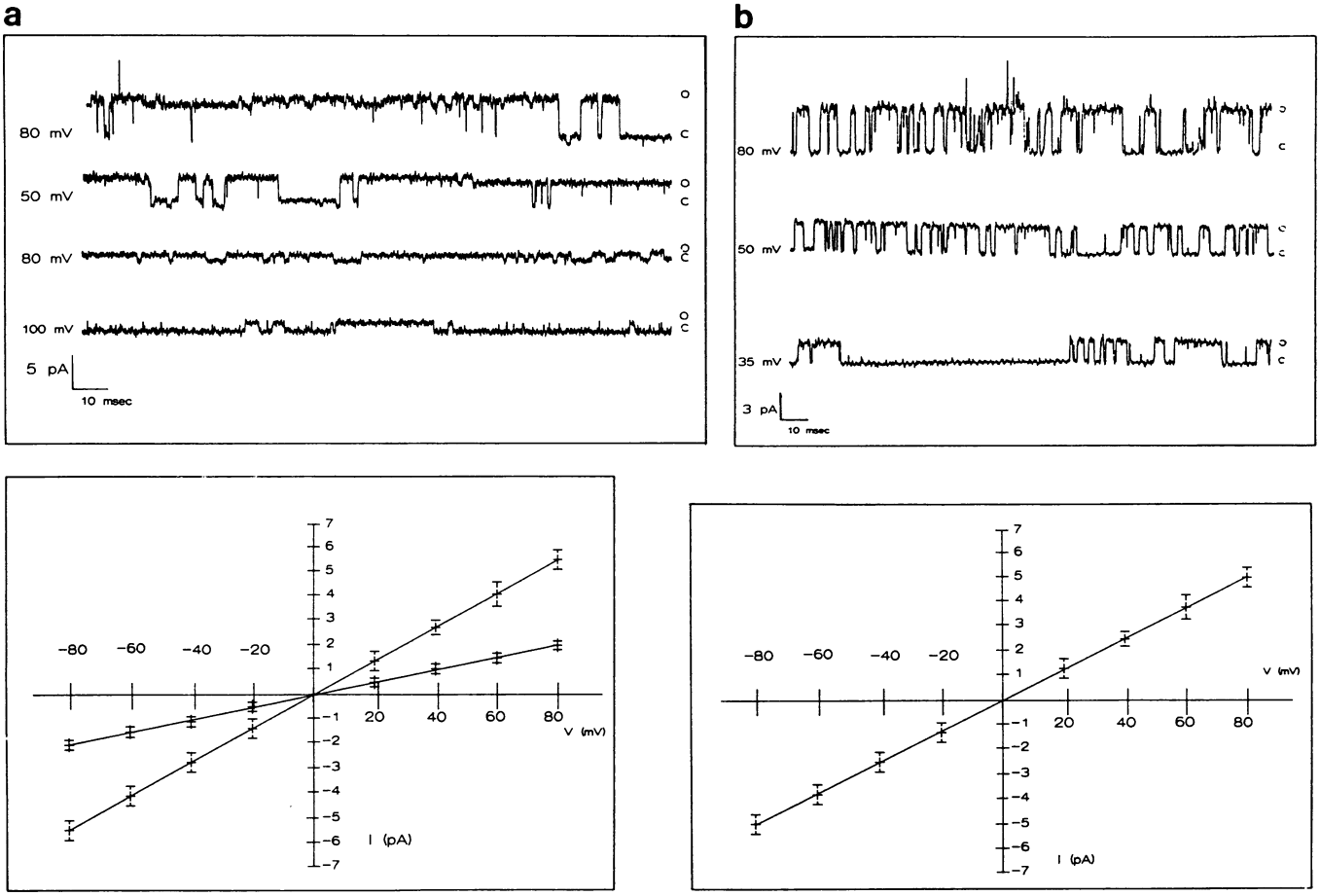

Figure 2. Recordings of channel activity and corresponding current voltage curves. (a) *P. maritima* grown without NaCl, inside out patch in a symmetrical solution containing 100 mM KCl, 10 mM Mes/Tris (pH 6.9), 0.1 mM $CaCl_2$. Two different channels can be distinguished, 68 ± 4 and 25 ± 2 pS. (b) *P. media* grown without NaCl, one type of channel with a conductance of 62 ± 3 pS can be distinguished. Membrane configuration and solution were as in (a).

currents were a summation of single channel currents through the 60 to 70 pS channels. Using the Goldman equation, a P_{K^+}/P_{Cl^-} of 5 was calculated from the ion activities and the reversal potential.

Effect of NaCl during Growth on Single Channel Characteristics

It is well known that high levels of NaCl in the growth medium cause a severe growth reduction of the salt sensitive *P. media*, while *P. maritima* can withstand much higher concentrations (2, 3, 14, 20). In the next series of experiments the effect of NaCl during growth, 25 and 100 mM, was tested on tonoplast channel behavior. The addition of 25 mM NaCl reduced the growth and survival of *P. media* and had essentially no effect on *P. maritima*. Addition of 100 mM NaCl strongly reduced growth of *P. media* and also reduced growth of *P. maritima*. For the patch clamp experiments, only those plants, grown on NaCl, were used that still appeared healthy.

Single channel conductances were determined, using excised patches from both species, grown at different NaCl levels. The results are summarised in Table I. As will be discussed in a next paragraph, in both species the application of a salt stress induced a severe decrease in the open probability. For this reason, most conductance and permeability parameters for plants grown on 100 mM NaCl could not be determined. Nevertheless, it seems quite clear that single channel conductances and selectivities were not affected by the different growth conditions.

Single Channel Kinetics

An important characteristic of a particular channel is its open time distribution. Figure 4a gives the open time distribution of the 60 to 70 pS channel in an inside out patch from *P. media*, grown without NaCl. The data were sampled at 100 mV, vacuolar side negative, over a period of 100 s. The mean open time was 2.3 ms, the total open time was 20% of the sample period. Figure 4b shows an example of the open time distribution of the same channel in an inside out patch from *P. media*, grown on 25 mM NaCl. The mean open time was 2.1 ms, the total open time was 27% of the sample period. Comparable results were obtained with tonoplast preparations from *P. maritima*. Obviously, the open time distribution and total open time during a burst of channel activity were not affected by the applied salt stress. For the same reason as

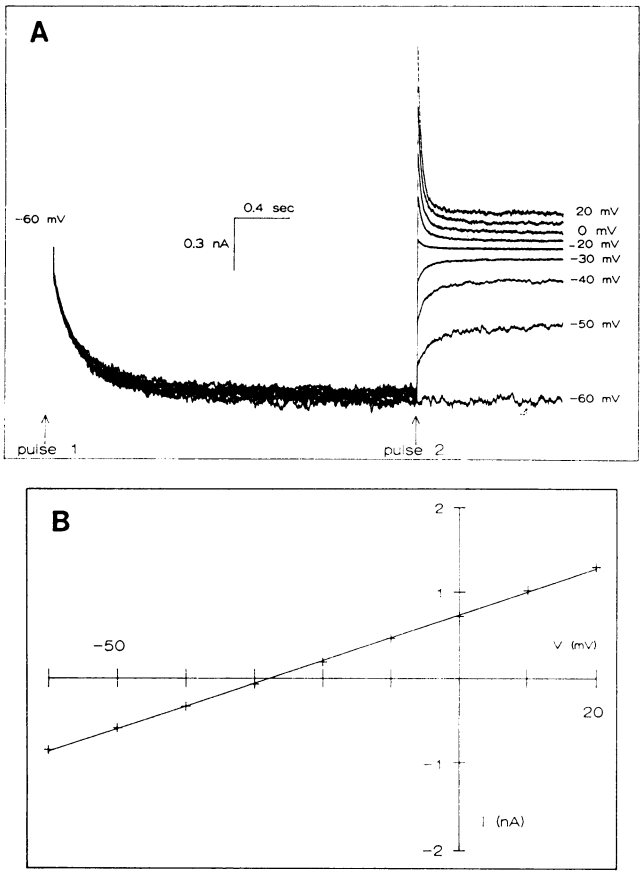

Figure 3. Double pulse experiment to determine cation over anion selectivity. (a) A *P. media* vacuole in the whole vacuole configuration was clamped at −60 mV for 5 s to induce channel opening (pulse 1). After channel opening, a set of second pulses was given which changed from −60 to 20 mV in 10 mV steps. From the tail currents after the second pulse, the reversal potential was calculated. Pipette solution: 100 mM KCl, 10 mM Mes/Tris (pH 6.9), 0.1 mM $CaCl_2$. Bath solution: 10 mM KCl, 10 mM Mes/Tris, 0.1 mM $CaCl_2$. (b) Current-voltage graph of the tail currents at the start of the second pulse. The reversal potential is −27 mV.

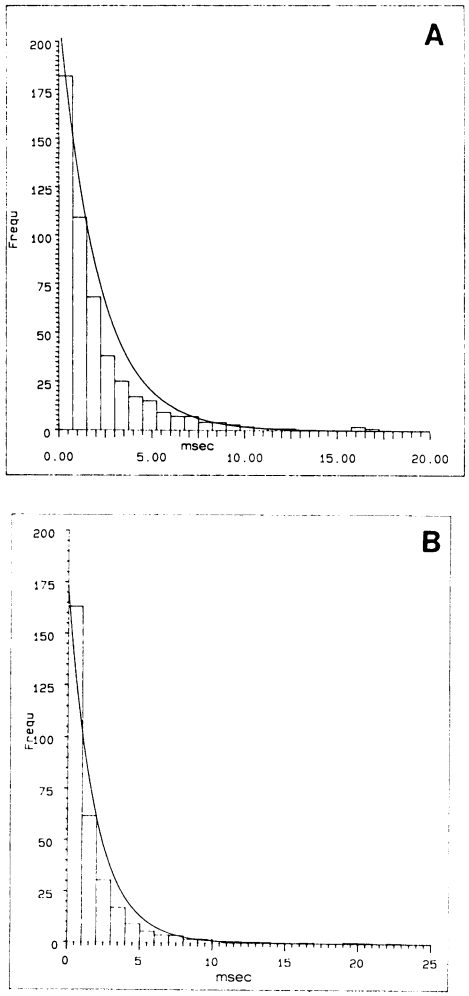

Figure 4. Open time distribution of the 62 pS channel in *P. media*: (a) *P. media* grown without NaCl. Inside out patch. A single exponential was fitted giving a mean open time of 2.3 ms. The holding potential was 100 mV, vacuolar side negative. (b) *P. media* grown in the presence of 25 mM NaCl. Mean open time was 2.1 ms at 100 mV. Solutions were as given in Figure 2.

Table I. *Single Channel Conductance in P. media and P. maritima*

Excised patches in symmetrical 100 mM KCl or NaCl solutions were used to measure single channel conductance. Plants were grown with 0, 25, or 100 mM NaCl supplemented to the growth medium. Data are expressed in pS ± SD.

Species	NaCl Concn	Single Channel Conductances	
		100/100 mM KCl	100/100 mM NaCl
	mM	*pS ± SD*	
P. media	0	62 ± 3	65 ± 4
	25	59 ± 3	64 ± 4
	100	60 ± 2	ND[a]
P. maritima	0	68 ± 4, 25 ± 2	70 ± 4, 25 ± 2
	25	63 ± 4, 26 ± 2	67 ± 4, ND
	100	ND	ND

[a] Not determined.

discussed above, there was not enough channel activity in patches from plants grown on 100 mM NaCl to produce reliable open time distributions.

Channel activity appeared in bursts. The frequency of the bursts was drastically reduced by the applied salt stress during growth. This resulted in a decreased open probability, as evidenced by the next series of experiments.

To study the effect of NaCl on the open probability, a large number of experiments was performed on vacuole preparations of both species, grown with 0, 25, or 100 mM NaCl. Vacuole attached and excised patches were monitored for channel activity. The results, presented in Table II, show that channel activity was very much reduced by the applied NaCl stress, as the result of a reduced frequency of bursts.

Effect of NaCl during Growth on Whole Vacuole Currents

Lack of single channel activity, when plants were grown in the presence of NaCl, was reflected in the whole vacuole

Table II. *Salt Stress Induced Decrease in Channel Activity*

Vacuole attached and excised patches were monitored for channel activity. If within 15 min more than 1 s activity occurred, experiments were regarded successful. Successful experiments are given as a percentage of the total number of experiments.

Species	NaCl Concn	No. of Experiments	Channel Activity
	mM		%
P. media	0	64	22
	25	51	17
	100	48	2
P. maritima	0	81	26
	25	72	13
	100	53	—

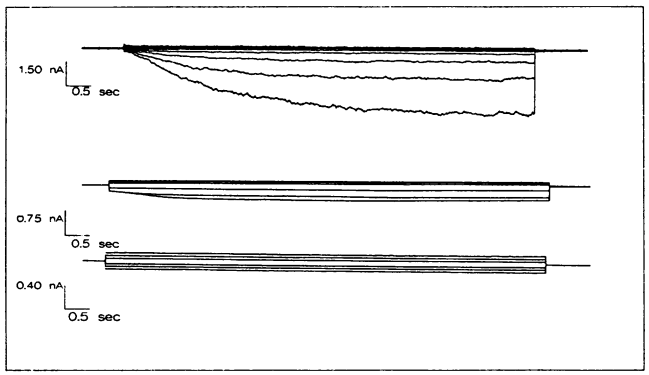

Figure 5. Whole vacuole currents. Vacuoles were clamped at 0 mV and the voltage was stepped from −60 to 60 mV. Solutions were as given in Figure 2. Upper trace: *P. media* grown without NaCl. Vacuole with 62 pS single channel activity. Middle trace: *P. maritima* grown on 25 mM NaCl. Vacuole with reduced channel activity. Lower trace: *P. maritima* grown on 100 mM NaCl. Vacuole without single channel activity.

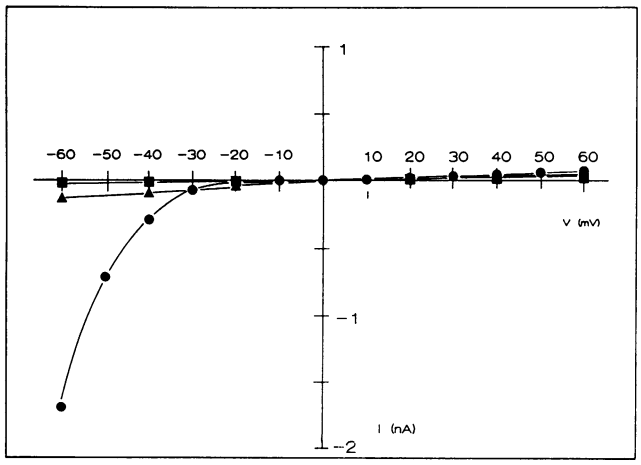

Figure 6. Current-voltage curves corresponding with Figure 5. After formation of the whole vacuole configuration, often small leak currents appeared. No correction was made for these ohmic leak currents. (●), Whole vacuole current in *P. media* grown without NaCl; (▲), whole vacuole current in *P. maritima* grown on 25 mM NaCl; (■), whole vacuole current in *P. maritima* grown on 100 mM NaCl.

current voltage relationship (Figs. 5 and 6). The upper trace of Figure 5, obtained with vacuoles of *P. media* grown in the absence of NaCl, shows that the 60 to 70 pS channels were slowly activated, and that inward rectification occurred. This has also been shown for tonoplasts of other species (1, 11–13).

Steady state currents were reached after about 3 s and were used for the current voltage curves in Figure 6. The lower traces of Figure 5 were derived from *P. maritima*, grown at 25 (middle trace) and 100 mM NaCl (lower trace). The strongly reduced channel activity is apparent here. Slowly activated currents disappeared, leaving a small rectification or no rectification at all (Fig. 5, middle and lower traces, and Fig. 6).

DISCUSSION

Earlier studies showed that *P. maritima* and *P. media* differ in Na^+ transport characteristics (2, 3, 14, 20). Since root vacuoles function as storage tissue, they form a buffer against a high influx of Na^+ (5, 8). The tonoplast is likely to play an important role in the selective sequestering of these ions. It was therefore expected that tonoplast single channel and whole vacuole current characteristics would differ between the species. It was also expected that the single channel characteristics might be affected by salt stress.

The most abundant channel in both *P. maritima* and *P. media* root tonoplast was a 60 to 70 pS channel which also is present in the tonoplast membranes of several other plant species (1, 11, 12). It was rather nonselective ($P_{K^+} = P_{Na^+} = 5P_{Cl^-}$), its gating was voltage dependent (Fig. 2), and it needed a relatively high Ca^{2+} concentration, 1 μM, on the cytoplasmic side. Inward rectification was observed in the whole vacuole configuration (Fig. 5). A smaller channel of 25 pS was present in *P. maritima* (Fig. 2), but with a lower frequency. Gating characteristics and Ca^{2+} dependence of this channel resembled those of the 60 to 70 pS channel. This observation was in contrast to the 'fast activated vacuolar' 30 to 40 pS channel, found in sugarbeet root vacuoles. These channels only showed activity at Ca^{2+} concentrations below 1 μM (13).

When plants were grown on 25 or 100 mM NaCl, selectivities and single channel conductances remained the same (Table I). The open probability, however, severely decreased (Table II). This behavior was present in vacuole attached, excised patch and whole vacuole configurations (Fig. 5).

The reduction in channel activity seems to be a physiological response. Very short bursts of channel activity were sometimes seen between periods, up to 30 min, without activity. This indicates that channels were still operational. During a burst of activity, the open time distribution and total open time were not affected by salt stress (Fig. 4), but the frequency of bursts was reduced. This reduction in open probability also resulted in a change of the current voltage relationship as measured in the whole vacuole configuration; when plants were grown in NaCl, the tonoplast conductance was low and rectification was less or disappeared.

ATP-dependent inward currents could be recorded in vacuoles showing channel activity, as well as in vacuoles with closed channels. This shows that also the latter group of vacuoles was still functioning (Fig. 1).

Although *P. maritima* and *P. media* differ in ion uptake and distribution, there was a great similarity in tonoplast channel properties. Ion selectivity, single channel conductance, and gating characteristics were comparable. Opening probability of the channels, in both species, was strongly reduced when the plants were grown on NaCl. Keeping these channels closed, also the 25 pS channel, may avoid the entrance of Na^+ into the cytoplasm, after storage in the vacuole. Assuming a 30 mV tonoplast potential *in vivo*, accumulation of Na^+ in the vacuole would be impossible when the channels were open. There is increasing evidence that Na^+ accumulation is mediated by H^+/Na^+ antiport (6, 7, 15). When the Na^+/H^+ antiport system is driven by the maximum ATPase current observed here (about 50 pA/vacuole), this current could not compensate the outward Na^+ currents through the open channels, which at least account for 100 to 200 pA.

Gating of the channels appeared at nonphysiological potentials and Ca^{2+} concentrations. The cytoplasmic Ca^{2+} concentration is usually far below 1 μM, although, as a result of hormone stimulation, it may temporarily rise to 1 μM (4). *In vivo*, the vacuolar potential is generally assumed to be slightly positive, 20 to 30 mV, with respect to the cytoplasm. Channel gating only occurred at rather positive potentials at the cytoplamic side. This would imply that the channels we observed are closed most of the time *in vivo*. In our opinion, the biological function of this kind of 'sleeping' channel seems hard to understand, unless it is assumed that during the preparation of vacuoles, some kind of regulating factor was lost, resulting in the requirement of rather unphysiological conditions to activate these channels.

Research about regulation of plant membrane channels has just started and it seems imperative to assume other gating mechanisms, besides voltage and cytoplasmic Ca^{2+}, in order to explain channel opening under more physiological conditions. Salt stress seems to induce such a regulating factor which prevents channels from opening when they normally would. This factor seems to be tightly bound to the membrane since bath perfusion could not delete its effect.

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